Journal of Medical Virology 49:87–90 (1996)

Absence of Extensive Genetic Heterogeneity of Hepatitis C Virus in Antibody-Negative Chronic Hepatitis C

J.H. Kao, P.J. Chen, P.M. Yang, M.Y. Lai, T.H. Wang, and D.S. Chen

Department of Internal Medicine (J.H.K., P.M.Y., T.H.W., D.S.C.); Graduate Institute of Clinical Medicine (P.J.C., M.Y.L.), and Hepatitis Research Center (D.S.C.), National Taiwan University Hospital, Taipei, Taiwan

Hepatitis C virus (HCV) carriers usually have antibodies to HCV; however, there are viremic individuals without these antibodies. To investigate whether variations of the viral genome are responsible for this discrepancy, the nucleotide and deduced amino acid sequences of HCV capsid and nonstructural regions obtained from 15 viremic patients were examined. These 15 patients were infected with type 1b HCV, and 10 did not have antibody to HCV assayed with second-generation tests. The nucleotide homology of the 5 seropositive and 10 seronegative patients with the HCV prototype sequence were 91.6% and 91.9%, respectively, in the capsid region. There was no apparent difference in the deduced amino acid sequences between the two groups of patients studied (94% vs. 95%). The nucleotide and amino acid sequences of a part of the nonstructural region 3 also showed similar results. These findings suggest that absence of antibodies against both capsid and nonstructural peptides in HCV carriers is not caused by genetic heterogeneity of the viral epitopes.

KEY WORDS: HCV EIA, RT-PCR, chronic hepatitis C, genetic variation

INTRODUCTION

With the molecular cloning of viral genome and subsequent development of serologic assays (first and second-generation EIAs) to detect antibodies [Choo et al., 1989; Kuo et al., 1989], hepatitis C virus (HCV) has been shown to be the major cause of chronic non-A, non-B hepatitis worldwide [Houghton et al., 1991; Nakatsuji et al., 1992; Chien et al., 1993]. However, there still exists a significant proportion of HCV-infected patients seronegative for anti-HCV by sensitive second-generation assays that contain the highly conserved capsid antigen [Alter et al., 1992; Sugitani et al., 1992]. Immunological incompetence but not variations of major epitopes (5-1-1) of C100-3 has been suggested for viremic patients seronegative by the first-generation assay [Hosoda et al., 1992]. A previous study also implicates the important role of immune regulation in the production of anti-C100-3 [Tsai et al., 1994]. By contrast, a recent report indicated that the sensitivity of first-generation assay is influenced by the heterogeneity in C100-3 sequences of different genotypes, while antibodies to capsid protein are detectable in all of their patients with viremia, irrespective of genotypes [Nagayama et al., 1993]. These controversial observations based on the first-generation assays prompted us to investigate further the possible mechanisms in those with HCV viremia but are repeatedly negative for antibodies against capsid and nonstructural antigens ("second-generation assay"). To examine whether the extensive genetic heterogeneity of HCV influences the sensitivity and specificity of serological tests used currently for diagnosis of HCV infection, the viral nucleotide and deduced amino acid sequences of the immunodominant capsid region as well as a part of the nonstructural region 3 (NS3) between anti-HCV-positive and negative patients were examined and compared.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 15 out of 206 patients with chronic non-A, non-B hepatitis seen in the gastroenterological clinic of the National Taiwan University Hospital. Among them, second-generation tests for anti-HCV were reactive in 179 (86.8%). These 15 patients (10 men, 5 women; mean age: 45.3 ± 6.6 years) were chosen because all were viremic for type 1b HCV, 5 were positive and 10 repeatedly negative for anti-HCV. All were seronegative for HBsAg and without markers of autoimmune hepatitis including antinuclear antibodies, antimitochondrial antibodies and anti-smooth muscle antibodies. None had a history of renal dialysis, alcoholism (>50 g/day), intravenous drug abuse, homosexuality,

Accepted for publication January 10, 1996.

Address reprint requests to Dr. D.S. Chen, Hepatitis Research Center, National Taiwan University Hospital, 1 Chang-Te St., Taipei, Taiwan 100.

TABLE I. Sequences of Oligonucleotide Primers for Amplification of Hepatitis C Virus Capsid Region

Primer number[a] (5′—>3′)	Nucleotide sequence	Position[b]
C3s	CGAAAGGCCTTGTGGTACTG	−70–−51
186a	ATGTACCCCATGAGGTCGGC	410–391
C5s	GATAGGGTGCTTGCGAGTGC	−47–−28
261a	GGGTGTCGATGACTTTACCC	376–357

[a]s, sense; a, antisense.
[b]Based on the Taiwanese HCV genome [Chen et al., 1992].

or hepatotoxic drug intake. Metabolic liver disease including Wilson's disease, hemochromatosis or α-1 antitrypsin deficiency were excluded by clinical and laboratory data. Chronic liver disease was defined by elevation of serum alanine aminotransferase (ALT) level more than twice the upper normal limit of normal range for longer than 6 months. No antivirals or immunomodulatory agents were given to these patients. The clinical and histological features between the two groups were comparable as described previously [Kao et al., 1996]. The serum samples taken from each patient were stored at −70°C until used.

Anti-HCV Test

All serum samples were assayed by a second-generation EIA (Abbott Laboratories, North Chicago, IL) that contains multiple antigens of C22-3 (119 aa, capsid), C33c (266 aa, NS3) and C100-3 (363 aa, NS4) to detect antibodies against HCV [Chien et al., 1993; Nagayama et al., 1993]. The manufacturer's instructions were followed.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay

Serum HCV RNA was detected by RT-PCR with different primer pairs to amplify three different regions (5′ untranslated region [5UT], capsid region, and nonstructural region 3 [NS3]) of the HCV genome [Kao et al., 1992], and the genotype of HCV was determined by a PCR typing assay [Kao et al., 1994]. The methods of RNA extraction, reverse transcription and PCR conditions as well as the two sets of nested primers deduced from 5UT and NS3 were the same as reported previously [Kao et al., 1992]. The oligonucleotide primers used to amplify the capsid region (C22-3) are shown in Table I. Samples from healthy persons and reagents without DNA were used as negative controls. Data were included only when all of the controls were negative. Each sample was tested in at least two separate runs.

Direct Sequencing of PCR Products

The amplified DNA from capsid and NS3 regions were sequenced directly by using fluorescence-labeled primers with a 373A Sequencer (Applied Biosystems, Foster City, CA). Sequencing conditions were specified in the protocol for the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The primer pair C5s as well as 261a (Table I) and an internal primer (5′-TACCTTCACCATTGAGA-3′) were used as sequencing primers for capsid and NS3 regions, respectively.

RESULTS

Direct Sequencing of the Capsid Region After PCR Amplification

The nucleotide sequences of the gene encoding C22-3 corresponding to positions 4 to 360 (357 nucleotides, 119 amino acids) of the prototype HCV cDNA sequence (HCV-1) [Choo et al., 1991; Nagayama et al., 1993] were determined in the serum HCV RNA of all 15 patients. The homology of nucleotide sequences between each patient and the prototype ranged from 90.6% to 92.5% in this region. The homology between 5 seropositive and 10 seronegative patients with the HCV prototype were 91.6% and 91.9%, respectively.

Of 5,355 nucleotides sequenced in the 15 samples, 446 (8.3%) nucleotides were different from those of the prototype and 67.9% (303 nucleotides) of the changes were located in the third position of each codon.

Amino Acid Sequence of the Capsid Region

The 119 deduced amino acid sequences of the 5 seropositive and 10 seronegative patients are shown in Figure 1. The homology between each sequence and that of the prototype ranged from 93% to 97% [Choo et al., 1991]. However, they were similar to each other and the homology of each sequence and that of the Taiwanese HCV genome was from 93% to 97% [Chen et al., 1992]. The peptide sequences in anti-HCV-positive and negative patients had an average homology of 94% and 95% to that of the prototype and a higher average homology of 94.6% and 95.5% to that of the Taiwanese HCV genome (Fig. 1). There existed 5 positions marked by asterisks within the capsid polypeptide, where deduced amino acid was different frequently from that of the prototype (Fig. 1). The pattern of the substitution was similar in each position in these Taiwanese patients but amino acid sequences specific for anti-HCV-positive or negative patients were not documented.

Amino Acid Sequence of NS3

PCR products from the NS3 region were partially sequenced and the deduced amino acid sequences of anti-HCV-positive and negative patients had an average homology of 89% and 86.5% to that of the prototype and a higher homology of 95.7% and 93% to that of the Taiwanese HCV genome, respectively [Choo et al., 1991; Chen et al., 1992].

DISCUSSION

The genome organization of HCV consists of 5′ and 3′ untranslated regions (5UT and 3UT) flanking the virus structural (capsid, envelope) and envelope region 2/ nonstructural region 1 (E2/NS1) and nonstructural regions 2 to 5 (NS2–NS5) [Choo et al., 1991; Houghton et al., 1991; Chen et al., 1992]. Although isolates cloned from different geographic areas show significant sequence divergence [Houghton et al., 1991], the capsid

A

```
          1       * *
HCV-1: MSTNPKPQKKNKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATR
HCV-T: ----G---R-T-----------------------------------------
 1.    --------R-TT----------------------------------------
 2.    --------R-T-----------------------------------------
 3.    --------R-T-----------------------------------------
 4.    ---D----R-T-----------------------------------------
 5.    --------R-T-----------------------------------------

                                  *                *
HCV-1: KTSERSQPRGRRQPIPKARRPEGRTWAQPGYPWPLYGNEGCGWAGWLLSP
HCV-T: ------------------Q----A---------------L------V--
 1.    ----------------------A---------------L-----F---
 2.    ------K----------D------A---------------L---------
 3.    ----------------------V---------------LL----F---
 4.    --T--P----------------A---------------L---------
 5.    --------------------V--A--------S-------E----F---
```

		Homology with:	
	120	HCV-1	HCV-T
HCV-1:	RGSRPSWGPTDPRRRSRNLG	-	93%
HCV-T:	-----N----------	93%	-
1.	----------	95%	95%
2.	-----N----------	94%	96%
3.	-----N----------	94%	95%
4.	----------	94%	94%
5.	-----N----------	93%	93%
	Average	**94.0%**	**94.6%**

B

```
          1       * *
HCV-1: MSTNPKPQKKNKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATR
HCV-T: ----G---R-T-----------------------------------------
 1.    --------R-T-----------------------------------------
 2.    --------R-TT----------------------------------------
 3.    --------R-T-----------------------------------------
 4.    --------R-TT----------------------------------------
 5.    --------R-T-----------------------------------------
 6.    ----------TT----------------------------------------
 7.    --------R-T-----------------------------------------
 8.    --------R-T-----------------------------------------
 9.    --------R-T-----------------------------------------
10.    --------R-T-----------------------------------------

                                  *                *
HCV-1: KTSERSQPRGRRQPIPKARRPEGRTWAQPGYPWPLYGNEGCGWAGWLLSP
HCV-T: ------------------Q----A---------------L------V--
 1.    ------------------P----A-------R-------L-----F---
 2.    ----------------------A-------R-------L-----V---
 3.    ----------------------A---------------L---------
 4.    --------------------T---D---------------LE--------
 5.    ----------------------A---------------L--------T
 6.    ----------------------A---------------L------F--
 7.    ----------G-----------A---------------LE--------
 8.    ----------------------A---------------L---------
 9.    ----------------------A---------------L---------
10.    -----T----------------A---------------L---------
```

		Homology with:	
	120	HCV-1	HCV-T
HCV-1:	RGSRPSWGPTDPRRRSRNLG	-	93%
HCV-T:	-----N----------	93%	-
1.	-----N----------	93%	96%
2.	----------	94%	94%
3.	----------	97%	97%
4.	-----N----------	93%	94%
5.	----------	96%	96%
6.	----------	96%	94%
7.	-----D----------	94%	96%
8.	----------	97%	97%
9.	H--------I----------	95%	95%
10.	-----D----------	95%	96%
	Average	**95.0%**	**95.5%**

Fig. 1. Amino acid sequences of the capsid region of hepatitis C virus (HCV) isolates from **(A)** 5 anti-HCV-positive patients and **(B)** 10 anti-HCV-negative patients by a second-generation EIA test (Abbott) are compared with the American (HCV-1) and the Taiwanese (HCV-T) type of sequences [Choo et al., 1991; Chen et al., 1992]. The number on the left represents each individual patient. Single-letter amino acid code is used. Dashes represent amino acid sequences identical to the HCV prototype and asterisks indicate the positions where amino acid substitution occurred frequently.

region contains a highly conserved sequence that elicits most global reactivity to antibodies against HCV [Nakatsuji et al., 1992; Chien et al., 1993; Nagayama et al., 1993]. The putative coding region for the capsid starts from the first amino acid to amino acid 190 of the HCV open reading frame [Choo et al., 1991; Houghton et al., 1991; Chen et al., 1992], and the most immunodominant epitopes have been localized exclusively in the amino-terminal 74 residues [Nasoff et al., 1991]. The capsid peptide, synthetic or recombinantly expressed, is therefore incorporated universally in current multispecific second-generation immunoassays for detecting anti-HCV antibodies [Nakatsuji et al., 1992; Chien et al., 1993; Nagayama et al., 1993]. However, several recent reports indicated that there exists a small but significant proportion (10% to 30%) of viremic individuals repeatedly seronegative by current second-generation anti-HCV assays [Alter et al., 1992; Sugitani et al., 1992]. Recent data, by using the second-generation assay, showed consistently that 8% of HCV-infected patients with chronic liver disease were persistently negative for anti-HCV [Kao et al., 1996]. We therefore sequenced the capsid and NS3 regions of the HCV genome in these viremic but seronegative patients to explore the possible viral mechanisms involved.

The homology in nucleotide sequences of the capsid between our patients and the prototype was 90.6–92.5%, and the homology in amino acid sequences was as high as 93–97%. These data were consistent with the previous report that the homology in nucleotide sequences between the Taiwanese and the American HCV genomes in this region was 88.7%, and the homology in amino acid sequences was 93.7% [Chen et al., 1992]. The amino acid sequence homology between our patients and the Taiwanese type was 93–97%, and these differences can be regarded as sequence variations of the Taiwanese strains. As to the NS3 region, the homology in amino acid sequences between our patients and the prototype ranged from 86.5% to 89% which was comparable to our previous data of 92.1% [Chen et al., 1992].

The nucleotide and the deduced amino acid sequences of HCV RNA obtained from 5 anti-HCV-positive and 10 negative patients were examined and significant differences in the homology of the nucleotide and deduced amino acid sequence (capsid and NS3) between anti-HCV-positive and negative patients were not found. The similarity of capsid and NS3 in both groups indicated that the seronegativity was not caused by variations of these immunodominant epitopes. For HCV sequences of 5-1-1 (a major epitope of C100-3), Hosoda et al. [1992] found that the homology in nucleotide and deduced amino acid sequences between anti-C100-3-positive and negative patients is actually comparable. In contrast, Nagayama et al. [1993] reported that the sensitivity of detectable anti-C100-3 is genotype-dependent while anti-C22-3 is not. Meantime, it was found using an in vitro system that removal of CD8+ T cells from peripheral blood mononuclear cells (PBMC) could increase the levels of anti-C100-3 secretion by PBMC of anti-HCV-seronegative patients [Tsai et al., 1994]. Taking previous

data and the present results together, it is clear that the absence of antibodies against recombinant antigens in HCV-infected individuals is not caused by extensive mutations of the HCV genome but by the incompetent host immunity. Whether CD8+ T cells contribute to the regulation of specific antibody production in such patients requires further studies.

ACKNOWLEDGMENTS

The study was supported by grants from the Department of Health, and the National Science Council (NSC85-2331-B-002-178), Executive Yuan, ROC.

REFERENCES

Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Gerber MA, Sampliner RE, Meeks EL, Beach MJ (1992): The natural history of community-acquired hepatitis C in the United States. New England Journal of Medicine 327: 1899–1905.

Chen PJ, Lin MH, Tai KF, Liu PC, Lin CJ, Chen DS (1992): The Taiwanese hepatitic C virus genome: Sequence determination and mapping the 5′ termini of viral genomic and antigenomic RNA. Virology 188:102–113.

Chien DY, Choo QL, Tabrizi A, Kuo C, McFarland J, Berger K, Lee C, Shuster JR, Nguyen T, Moyer DL, Tong M, Furuta S, Omata M, Fong CT, Tegtmeier G, Alter H, Schiff E, Jeffers L, Houghton M, Kuo G (1993): Use of recombinant HCV antigen in the serodiagnosis of hepatitis C virus infection: Significant improvement in HCV antibody detection as compared with the first generation HCV C100-3 ELISA and the synthetic peptide EIA tests. Journal of Gastroenterology and Hepatology 8:S33–39.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.

Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic organization and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences USA 88:2451–2455.

Hosoda K, Omata M, Yokosuka O, Kato N, Ohto M (1992): Nucleotide sequences of 5-1-1 of hepatitis C virus in patients with chronic liver disease. Gastroenterology 102:1039–1043.

Houghton M, Weiner A, Han J, Kuo G, Choo QL (1991): Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology 14:381–388.

Kao JH, Chen PJ, Yang PM, Lai MY, Sheu JC, Wang TH, Chen DS (1992): Intrafamilial transmission of hepatitis C virus: The important role of infections between spouses. Journal of Infectious Diseases 166:900–903.

Kao JH, Chen PJ, Lai MY, Yang PM, Sheu JC, Wang TH, Chen DS (1994): Mixed infections of hepatitis C virus as a factor in acute exacerbation of chronic type C hepatitis. Journal of Infectious Diseases 170:1128–1133.

Kao JH, Lai MY, Huang YT, Yang PM, Chen PJ, Sheu JC, Wang TH, Hsu HC, Chen DS (1996): Chronic hepatitis C without anti-hepatitis C antibodies by second generation assay: A clinicopathologic study and demonstration of the usefulness of third generation assay. Digestive Diseases and Sciences 41:161–165.

Kuo G, Choo QL, Alter HJ, Gitnick GI, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Overby LR, Bradley DW, Houghton M (1989): An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364.

Nagayama R, Tsuda F, Okamoto H, Wang Y, Mitsui T, Tanaka T, Miyakawa Y, Mayumi M (1993): Genotype dependence of hepatitis C virus antibodies detectable by the first-generation enzyme-linked immunosorbent assay with C100-3 protein. Journal of Clinical Investigation 92:1529–1533.

Nakatsuji Y, Matsumoto A, Tanaka E, Ogata H, Kiyosawa K (1992): Detection of chronic hepatitis C virus infection by four diagnostic systems: First-generation and second-generation enzyme-linked immunosorbent assay, second-generation recombinant immunoblot assay and nested polymerase chain reaction analysis. Hepatology 16:300–305.

Nasoff MS, Zebedee SL, Inchauspe G, Prince AM (1991): Identification of an immunodominant epitope within the capsid protein of hepatitis C virus. Proceedings of the National Academy of Sciences USA 88:5462–5466.

Sugitani M, Inchauspe G, Shindo M, Prince AM (1992): Sensitivity of serological assays to identify blood donors with hepatitis C viraemia. Lancet 339:1018–1019.

Tsai SL, Chen PJ, Hwang LH, Kao JH, Huang JH, Chang TH, Chen DS (1994): Immune response to a hepatitis C virus nonstructural protein in chronic hepatitis C virus infection. Journal of Hepatology 21:403–411.